Faculty of Resource Science and Technology

Molecular Characterization and Similarity Relationship among Selected Kelampayan (*Neolamarckia cadamba*) Genotypes from Kelampayan Provenance Trial Plot I using ISSR Markers

Elias B. Haji Abu Bakar

Bachelor of Science with Honours

(Resource Biotechnology)

2010

Molecular Characterization and Similarity Relationship among Selected Kelampayan (*Neolamarckia cadamba*) Genotypes from Kelampayan Provenance Trial Plot I using ISSR Markers

Elias Haji Abu Bakar

A project Report submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2010

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr. Ho Wei Seng for his guidance, advice and support throughout the project. Furthermore, I would also like to appreciate and thank to Mr. Phui Seng Loi (MSc. Student), Ms. Lai Pei Sing (MSc. Student), and Ms. Tiong Shing Yiing (MSc. Student) for their guidance and dedication. There are also special thanks to Ms. Tchin Boon Ling (MSc. Student), Mr. Liew Kit Siong (MSc. Student), Ms. Grace Ting (MSc. Student) and all my friends for their assistance and kindness towards guidance me to do my project works. Finally, I would like to thanks to my family, especially my mother Mdm. Patimah Bt. Ahmad, for their moral support and encouragement through completing my project.

TABLE OF CONTENTS

ACKNOLEDGEMENT		I
TABLE OF CONTENTS		II
LIST OF TABLES		V
LIST OF FIGURES		VI
LIST OF ABBREVATIONS		IX
ABSTRACT AND ABSTRAK		1
CHAPTER 1	INTRODUCTION	2
CHAPTER 2	LITERATURE REVIEW	4
	2.1 *Neolamarckia cadamba* (Roxb.) Bosser	4
	2.2 Molecular marker	5
	2.3 Inter Simple Sequence Repeats (ISSR)	5
	2.4 Application of ISSR-PCR in *Rubiacea*e family	6
CHAPTER 3	MATERIALS AND METHODS	8
	3.1 Plant materials	8
	3.2 DNA isolation	8
	3.3 DNA purification	9
	3.4 DNA visualization	9
	3.5 DNA quantification	10
	3.6 ISSR-PCR optimization	10

3.6.1 Thermal cycling profile 10

3.6.2 DNA concentration 11

3.6.3 Optimum ISSR-PCR conditions 11

3.7 ISSR-PCR analysis 12

3.8 Agarose gel electrophoresis (AGE) and PCR products visualization 12

3.9 Data analysis 13

CHAPTER 4 RESULTS AND DISCUSSION 14

4.1 DNA isolation and purification 14

4.2 DNA quantification 15

4.3 ISSR-PCR optimization 16

4.3.1 Annealing temperature (T_a) 16

4.3.2 DNA concentration 18

4.3.3 Magnesium Chloride ($MgCl_2$) 20

4.4 ISSR-PCR analysis 20

4.5 Data analysis 24

4.5.1 Data scoring 24

4.5.2 Genetic diversity 27

4.5.3 Genetic relatedness 28

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS 39

REFERENCES 40

APPENDIX A 44

APPENDIX B 45

APPENDIX C 46

APPENDIX D 50

APPENDIX E 57

APPENDIX F 60

APPENDIX G 63

APPENDIX H 66

APPENDIX I 73

APPENDIX J 79

APPENDIX K 85

APPENDIX L 91

LIST OF TABLES

Table		Page
3.1	Details of ISSR markers.	11
3.2	PCR Mixture.	11
3.3	Thermal cycling profile for ISSR-PCR.	12
4.1	Spectrophotometer reading of the samples.	15
4.2	Samples of ISSR-PCR $(GTG)_6$, $(AC)_{10}$ and $(AG)_{10}$ primer amplifications for figure 4.9, 4.10 and 4.11.	22
4.3	Locus name and fragment size of ISSR (GTG_6), (AC_{10}) and (AG_{10}) primers.	24
4.4	Distribution of samples in the dendrogram cluster.	31
4.5	Tree origin according to lane in Block 1.	31

LIST OF FIGURES

Figure		Page
1.1	Kelampayan (*Neolamrackia cadamba*) leaves and Flower.	2
4.1	The electrophoresis of purified DNA on 0.8% agarose gel.	14
4.2	Electrophoresis of ISSR-PCR $(GTG)_6$ primer annealing temperature optimization products on a 1.5% Agarose gel.	16
4.3	Electrophoresis of ISSR-PCR $(AC)_{10}$ primer annealing temperature optimization products on a 1.5% Agarose gel.	17
4.4	Electrophoresis of ISSR-PCR $(AG)_{10}$ primer annealing temperature optimization products on a 1.5% Agarose gel.	17
4.5	Electrophoresis of ISSR-PCR $(GTG)_6$ primer DNA concentration optimization products on a 1.5% Agarose gel.	18
4.6	Electrophoresis of ISSR-PCR $(AC)_{10}$ primer DNA concentration optimization products on a 1.5% Agarose gel.	18
4.7	Electrophoresis of ISSR-PCR $(AG)_{10}$ primer DNA concentration optimization products on a 1.5% Agarose gel.	19

4.8	Electrophoresis of ISSR-PCR $(AC)_{10}$ primer $MgCl_2$ concentration optimization products on a 1.5% Agarose gel.	20
4.9	Electrophoresis of ISSR-PCR $(GTG)_6$ primer products on a 1.5% Agarose gel.	21
4.10	Electrophoresis of ISSR-PCR $(AC)_{10}$ primer products on a 1.5% Agarose gel.	21
4.11	Electrophoresis of ISSR-PCR $(AG)_{10}$ primer products on a 1.5% Agarose gel.	21
4.12	Graph $\log_{10}$ market fragment size versus band migrated distance.	26
4.13	UPGMA dendrogram showing relationship among *N. cadamba* trees in block I using $(GTG)_6$, $(AC)_{10}$ and $(AG)_{10}$ ISSR primers.	30
4.14	Neighbor-joining tree showing relationship among *N. cadamba* trees in block I using $(GTG)_6$, $(AC)_{10}$ and $(AG)_{10}$ ISSR primers.	32
4.15	UPGMA dendrogram showing relationship among *N. cadamba* trees in block I using $(GTG)_6$ ISSR primer.	33
4.16	Neighbor-joining tree showing relationship among *N. cadamba* trees in block I using $(GTG)_6$ ISSR primer.	34

4.17	UPGMA dendrogram showing relationship among *N. cadamba* trees in block I using $(AC)_{10}$ ISSR primer.	35
4.18	Neighbor-joining tree showing relationship among *N. cadamba* trees in block I using $(AC)_{10}$ ISSR primer.	36
4.19	UPGMA dendrogram showing relationship among *N. cadamba* trees in block I using $(AG)_{10}$ ISSR primer.	37
4.20	Neighbor-joining tree showing relationship among *N. cadamba* trees in block I using $(AG)_{10}$ ISSR primer.	38

LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260 nanometers
CTAB	Centytrimethylammonium Bromide
bp	base pair
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dsDNA	Double Stranded DNA
EDTA	Ethylene diaminetetra-acetic acid
EtBr	Ethidium Bromide
HCl	Hydrochloric acids
ISSR	Inter-simple Sequence repeats
ISSR-PCR	Inter-Simple Sequence Repeat-Polymerase Chain Reaction
Kb	Kilo base
mM	milimolar
NaCl	Sodium Chloride
ng/ml	Nanogram per milliliters
nm	Nanometers
NTSYS-pc	Numerical Taxonomy System for Windows

O.D.	Optical Density
PCR	Polymerase Chain Reactions
RAPD	Random Amplified Polymorphic DNA
RNase	Ribonuclease
Rpm	revolution per minutes
UPGMA	Unweighted Pair-Group Method with Arithmetrical Averages
V	Volt
µg/ml	microgram per milliliters
µL	microliters
µM	micromilliliters

Molecular characterization and similarity relationship among Selecetd Kelamapayan (*Neolamarckia cadamba*) Genotypes from Kelampayan provenance Trial Plot I using ISSR Markers

Elias Haji Abu Bakar

Resource Biotechnology Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Neolamarckia cadamba or Kelampayan is a fast growing timber species that has economical and medicinal value. Kelampayan is one of the species widely used for reforestation programme, so as high commercial value in plywood manufacturing and wound cleansing. Therefore, molecular characterization of this species is important to enable further species improvement. In this project, determination of genetic relatedness and diversity of kelampayan was accomplish using ISSR primers namely $(GTG)_6$, $(AC)_{10}$ and $(AG)_{10}$. Selected primers had successful amplified 64 loci with 73.44% of polymorphic in block I population. Shannon's diversity index of all three combined primes was reported to be 0.350. Molecular diversity based on Jaccard's similarity coefficient of 79 trees was in the ranged of 0.14 to 0.94. UPGMA dendrograms and neighbor joining tree were also constructed to show the genetic relationship of *N. cadamba* trees. In the outcome of the dendrograms reveals that, a total of four clusters were grouped for block 1 kelampayan trees indicating that *N. cadamba* trees are genetically diverse.

Keywords: Kelampayan (*Neolamarckia cadamba*), Inter Simple Sequence Repeat (ISSR) marker, Polymorphism, Genetic diversity.

ABSTRAK

Neolamarckia cadamba atau kelampayan ialah spesies balak yang cepat membesar serta mempunyai nilai ekomoni dan perubatan. Kelampayan telah digunakan secare meluas sebagai salah satu spesies bagi tujuan penghutanan semula, pembuatan papan lapis dan untuk membersihkan luka. Oleh itu, pencirian molekul bagi spesies ini adalah penting untuk membolehkan peningkatan kualiti spesies pada masa depan. Penentuan kepelbagaian genetik dan hubungan antara kelampayan telah dijalankan mengggunakan penanda ISSR yang dinamakan $(GTG)_6$, $(AC)_{10}$ dan $(AG)_{10}$. Penanda yang dipilih telah berjaya mengamplifikasi 64 lokus dengan 73.44% polimorfik di dalam populasi blok I. Index kepelbagaian Shannon dari gabungan ketiga-tiga penanda ialah 0.350. Kepelbagaian molekul berdasarkan pekali keserupaan Jaccard bagi 79 pokok berjarak dari 0.14 hingga 0.94. UPGMA dendrogram dan neighbor joining tree telah dibina bagi menunjukkan perhubungan genetik pokok N. cadamba. Dalam dendrogram, sejumlah empat kumpulan telah dikelaskan bagi blok 1 pokok kelampayan menunjukkan bahawa pokok N. cadamba adalah mempunyai kesamaan dan kepelbagaian dari aspek genetik.

Kata kunci: *Kelampayan (Neolamarckia cadamba), penanda Inter Simple sequence Repeat (ISSR), Polimorfikasi, Kepelbagaian genetik.*

CHAPTER 1

INTRODUCTION

Neolamarckia cadamba (Roxb.) Bosser from family *Rubiaceae* (Germsplan Resource Information Network, 1984) or Kelampayan is a fast growing timber species (Ismail *et al*., 1995) that has been used in the wood industry such as in making plywood and other light wooden products (Lim *et al*., 2005). This plant has various common names such as Laran in Indonesia, Kadam in India (USDA GRIN, 2009) and common burrflower-tree in Australia (James Cook University, 2009). This tree is considered native to Asian tropical country (China fro instance) and Temperate Asia country such as India, Cambodia, Indonesia, Malaysia and Papua New Guinea. It produces gold (yellow-orange) color flowers with globe like head and fragrance that attract bees, butterflies and birds (Dave, 2009).

Figure 1.1: Kelampayan(*N. Cadamba*) leaves and Flower.
(Adapted from http://cms.jcu.edu.au/discovernature/Neomarckia cadamba.htm)

Kelampayan grows well at swampy, moist area such as secondary forest by the riverbank (Chudnoff & Martin, 1984). Samples of this tree were collected from Kelampayan Provenance trial Plot Landeh which was located in swampy and hilly areas, suitable for the kelampayan growth and alike to its original habitat.

Kelampayan trees have been one of the important species to the timber industry, with necessity for the quality of this species to be maintained and preserved. Therefore, determination of similarity relationship among kelampayan from difference place is essential.

Molecular characterization is used to distinguish the relationship between closely related species. This method has come to popularity as it provides results that are more precise and accurate than physical observation method. Unlike morphological markers that are affected by epistatic effect and isozyme markers that relied on environment and post-translational modification, DNA markers are excluded from environmental factor and used for determination of genetic similarities among genotypes (Ozrenk *et al.*, 2010). ISSR-PCR technique been used in this project requires minute amount of starting materials and give dominant markers with high degree of polymorphism (Wu *et al*, 1994).

Genetic information of kelampayan is so far limited. The genetic information has increased-importancy since there are a lot of new species that have emerged as a result of natural mutation, cross pollination and propagation (Ozrenk *et al.*, 2010). Studies of kelampayan in other aspects such as seed improvement and pharmacological also rely on the availability and quality of genetic information.

The objectives of this project are to characterize kelampayan from different location via molecular aspect and to determine relationship similarity among kelampayan genotypes.

CHAPTER 2

LITERATURE REVIEW

2.1 *Neolamarckia cadamba* (Roxb.) Bosser

Neolamarckia cadamba or kelampayan has high economical value because of its wood properties and medicinal value. Kelampayan is a fast growing timber species (Phillips *et al*., 2002) with up to 10 meters straight bole (Ismail *et al*., 1995) that grows at wet lowland or riverbank (Ambujakshi *et al*., 2009). Kelampayan wood is soft, light and has smooth plane, which is easy to re-saw and cross cut. This species is suitable for light wood industry such as for making plywood, disposable chopstick and packing case (Timber Technology Centre, 1999).

The leaves and barks of kelampayan have been used for medicinal purpose and are believed to have analgesic characteristics (Patel *et al*., 2008). In addition, pheripheral analgesic activity of kelampayan can be applied as wound cleansing (Ambujakshi *et al*., 2009). A tribe in India has used kelampayan as paste for skin disease. Researchers have also been carried out to study its antimicrobial and antifungal activities on human skin (Umachigi *et al*., 2007). Furthermore, the aqueous extract of kelampayan was also found to reduce glucose level in blood and it was suspected to have related to diabetes medicine (Acharyya *et al*., 2010).

In 2008, kelampayan has been selected as one of the species for forest restoration by Sabah Forestry Department due to it fast growing characteristic and high wood quality to cope with deforestation. Restoration of forest is important to re-establish and enhanced the forest structure and diversity (Sabah Forestry Department, 2008).

2.2 Molecular Marker

Molecular marker has been one of the important methods that have large number of applications including genome analysis and fingerprinting. This method has also been constantly modified in order to enhance its utility and to produce good genetic information (Joshi *et al*., 1999).

Previously morphological characteristic was used for genetic diversity and similarity analysis, whereby the traits only represent a small portion of plant genome (Tanee *et al*., 2009). This conventional method was highly influenced by environmental factors and post-translational factor that limit the application and information obtained (Ozrenk *et al*., 2010). Molecular marker has been proven as a rapid and easy method for genomic analysis to generate greater amount of information.

Many PCRbased molecular markers have been constructed to overcome the limitation of certain methods and obtain desirable information from the genomic analysis. The examples of PCR-based molecular markers are Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR), and Amplified Fragment Length Polymorphism (AFLP). Since these methods are independent from environment factors, they have been widely used as an indicator of variation for genomic research (Tanee *et al*., 2009).

2.3 Inter Simple Sequence Repeats (ISSR)

Inter-Simple Sequence repeats (ISSR) have been extensively used for genomic analysis nowadays because it had proven as a powerful, rapid, simple reproducible and inexpensive method to obtain the genetic diversity information in many species (Kumar et al., 2008). ISSR markers are highly reproducible, do not require extensive knowledge of the target

sequence and give highly polymorphic fingerprints (Galvan *et al*., 2003). ISSR also come in abundance and highly disperse throughout the genome, making it an ideal marker for genetic mapping, populations study and genomic research (Hussein *et al*., 2008).

This method uses single primer of di-, tri- or tetra repeated nucleotides that is 15 to 20 nucleotides long with a 3' or'5 anchors sequence that target multiple loci (Pharmawati *et al*., 2004; Zietkiewickz *et al*., 1994). The popular repeat unit is tetra because there is no problem regarding the stutter region and replications slippage that can alter the length and constituent of sequence base pair. ISSR have been widely used for genetic diversity researches and cultivar identification (Bhattacharya et al., 2010; Pharmawati *et al*., 2004; Galvan *et al*., 2003). This technique also generates large number of markers by targeting and amplifying multiple loci, thus allowing screening of large number of samples at one time (Nagaraju *et al*., 2001).

ISSR markers have been reported to have successfully analyze the plant cultivars identification and diversity such as in strawberry germplasm (Kuras *et al*., 2004) and was also applied in interspecific polymorphism study in rice, tomato, and chickpea (Pharmawati *et al*., 2004). Despite that, this technique has good potential to be used as an alternative to RFLP technique that has been tested on poplar, roses, pea and hortensien (Hussein *et al*., 2008).

2.4 Application of ISSR-PCR in *Rubiaceae* family

There are wide application of ISSR-PCR marker in forestry conservation and agriculture. ISSR has been proven to be one of the important tools for conservation and plant improvement programme as information about targeted DNA sequences are not required

(Galvan *et al*., 2003). ISSR marker is suitable to be used on forest species that have not been extensively studied and with less genetic information.

ISSR-PCR technique has been widely applied on other species of Rubiaceae family, such as study on the genetic relationship of *Coffea* species (Ruas *et al*., 2003). The study of genetic relationship of different species was done between *Coffea* species and India *Psilanthus* (Kumar *et al*., 2008) using ISSR and RAPD molecular markers. The results gave high polymorphic banding patterns and reproducible for analysis.

In the study of phylogenetic and classification of *Nucleeae S.L* , ISSR technique data and morphological data was combined and subsequently altered the perspective and view of *Rubiaceae* family (coffee family) at all taxonomic level due to inconsistent subtribal and generic concept applied (Razafimandimbison and Bremer, 2002). This proved the usefulness of ISSR technique as a powerful approach to give high amount of information for further molecular study. Furthermore, combination of different data sets can be seen as one good problem solving skill to eliminate errors by one technique while reveals slight differences of resolution and support.

Application of ISSR markers on other plant species and family are for instance the relation identification and resolving of Canary Island *Tolpis* Species (Archibald *et al*., 2006) and population differentiation of common bean (Gonzalez *et al*., 2005). ISSR-PCR produced reproducible and high level of polymorphism band pattern that provides adequate information for further research work (Kumar *et al*., 2008).

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Materials

Kelampayan leaves were collected from the Kelampayan Provenance Trial Plot at Landeh Natural Reserve, Sarawak. A total of 83 samples were collected from the plot 1, (Appendix B).

3.2 DNA Isolation

The modified CTAB Doyle and Doyle (1978) method was performed to extract the total DNA from the leaves. 83 DNA samples were isolated using this method. CTAB extraction solution contained 2% (w/v) CTAB buffer, 100mM Tris-Cl at pH 6.0, 20mM EDTA at pH 8.0 and 1.4M NaCl and 2% (v/v) ß-mercaptoethanol was added into CTAB extraction solution and incubated at 65°C for 15 minutes before use.

1.0g of leaf tissue from each sample was ground into fine powder form by using mortar and pestle with the presence of liquid nitrogen. Then the pulverized sample was transferred into an eppendorf tube containing 600 ml pre-warmed CTAB extraction solution and mixed by inverting the tube. The solution was incubated at 65°C for 2 hours with occasional mixing. After that, 700µl of the solution was transferred into a 1.5 ml microcentrifuge tube and equal amount of chloroform: isoamyl alcohol (24:1) was added after the tube had cooled down to room temperature. The mixture was gently inverted for 25 times and centrifuged at 13000rpm for 10 minutes. The top aqueous phase was transferred into a new 1.5mL microcentrifuge tube and chloroform/isoamyl alcohol (CIA) extraction was repeated. Then, the supernatant was transferred into another new 1.5 ml

microcentrifuge tube and 2/3 volume of cold isopropanol was added to precipitate the DNA and incubate overnight at -20°C. Then the tube was centrifuged at 13000rpm for 10 minutes, the supernatant was poured off and 1ml of cold 70% ethanol was added and incubated for 1 hour to wash the DNA. After that, ethanol was aspirated from the tube by leaving it to air-dry at room temperature. The DNA pellet was then being dissolved in TE buffer (10mM Tris-HCL, 1 mM EDTA, pH 8.0) (Henry, 2001) prior to storage.

3.3 DNA purification

The DNA was purified using wizard genomic DNA purification Kit (Promega, USA). The resuspended DNA was topped up with ultra pure distilled water to 600 µL and 3 µL of 4 mg/mL RNase A was added to the solution. The solution was mixed and incubated at 37°C for 15 minutes. After that, 200 µL of protein Precipitate Solution was added and the solution was mixed vigorously. The solution was centrifuged at 13000 rpm for 3 minutes. The supernatant was then transferred into a new 1.5 mL microcentrifuge tube containing 600 µL of isopropanol, mixed and incubated at -20°C for 30 minutes. Then, the tube was centrifuged at 13000 rpm for 1 minute. Supernatant was decanted and 600 µL of 70% ethanol was added to wash the DNA pellet. Then, ethanol was aspirated and the DNA pellet was air-dried at room temperature. The DNA pellet was resuspended in sterile ultrapure water (volume depend on size of pallet) and stored at -20°C.

3.4 DNA Visualization

The DNA visualization performed using electrophoreses agarose gel (Stone, 2004) and 3µl of DNA mixed with 2µl of 1X loading dye (15% Ficoll, 120 mM EDTA, 0.25% xylene cyanol FF and 0.25% bromophenol blue). 3µl of λ *Hind* III was used as DNA markers (Promega, USA) and mixed with 2µl of loading dye loaded into the first well of the gel.

Agarose gel electrophoresis (AGE) was run at 50V for 1 hour. Then, the agarose gel was stained using ethidium bromide and destained in ddH_2O for 30 minutes without direct exposure to light. The gel photo was visualized and documented using Geliance 200 imaging system (Perkin Elmer).

3.5 DNA Quantification

For DNA quantification to take place, UV spectrophotometer (Perkin Elmer) was used. The DNA sample was diluted (1:10) with ultra pure water by adding 10µl DNA sample into 900µl sterile ultra pure water. Then the diluted DNA was transferred into a cuvette and the absorbance of the sample at 230nm, 260nm, and 280nm was read. The readings were then used to calculate the quantity of DNA using the formula:

$$[DNA] = A_{260} \text{ X } OD_{260} \text{ X dilution factor}$$

3.6 ISSR-PCR Optimization

3. 6.1 Thermal cycling profile

The optimization of annealing temperature for each primer is essential in order to produce high banding pattern. A total of 12 temperatures were tested in the range of 55°C-65°C. PCR optimization was performed according to Taguchi method (Weising *et al*., 2005) whereby predicted annealing temperature (T_a) of a primer is about ±5°C from the calculated temperature. The optimum thermal cycling profile for ISSR-PCR primer is shown in Table 3.3.

Primer concentrations tested were 0.2µM, 0.4µM, 0.6µM, 0.8µM and 1µM and varied depending on tendency of dimer forming and mispriming sites in templates. The magnesium chloride ($MgCl_2$) concentration that has been tested was 0.5mM, 1.5mM,

2.5mM (Weising *et al*., 2005). A total of 39 cycles was performed throughout optimization.

Table 3.1: Details of ISSR primers.

Primer name	Nucleotide sequence (5'-3')
$(GTG)_6$	GTG GTG GTG GTG GTG GTG
$(AC)_{10}$	ACA CAC ACA CAC ACA CAC AC
$(AG)_{10}$	AGA GAG AGA GAG AGA GAG AG

3.6.2 DNA Concentration

The DNA sample was diluted into a concentration series of 50ng/ml, 10ng/ml, 5ng/ml, 3ng/ml, 2ng/ml. Serial dilution was performed to obtain the clearest, reproducible and scorable DNA bands.

3.6.3 Optimum ISSR-PCR Condition

ISSR primers used were namely $(AC)_{10}$, $(AG)_{10}$ and $(GTG)_6$. PCR was carried out using Mastercycler Gradient PCR (eppendorf, Germany) machine.

Table 3.2: PCR Mixture.

Reagent	**Concentration**
1X Reaction buffer	2.5 µL
$MgCl_2$	50mM
dNTAs (dATP, dCTP, dTTP, dGTP)	2.0 mM
Taq DNA polymerase	0.5 unit
Primer	2.5 pmol/µl
DNA	1ng/µl

Table 3.3: Thermal cycling profile for ISSR-PCR primer.

Parameter	Temperature (°C)	Timing (min)	
Initial denaturation	94.0	2.0	
Denaturation	94.0	0.5	39 cycles
Annealing $(AC)_{10}$	57.8	0.5	
$(AG)_{10}$	56.6	0.5	
$(GTG)_6$	59.1	0.5	
Extension	72.0	1.0	
Final extension	72.0	10.0	

3.7 ISSR-PCR analysis.

The ISSR-PCR amplification of each DNA samples was performed based on the optimum PCR thermal cycling profile (Table 3.3) and PCR mixture (Table 3.2). An amplified product later was electrophoresed using 1.5% agarose gel with 1kb DNA ladder (Promega, USA).

3.8 Agarose gel PCR product visualization.

Two microliters of 6X loading dye (15% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol FF and 120 mM EDTA) was mixed with 7µl PCR product and loaded into 1.5% agarose gel. Then 1 kb DNA ladder (Promega, USA) was loaded into the first well in agarose gel. The AGE was run at 70V for 2 hours. After electrophoresis, the gel was stained using ethidium bromide and destained in distilled water for 30 minutes without direct exposure to light. Then, the gel with PCR products was visualized using UV transilluminator and Geliance 200 Imaging System (Perkin Elmer) was used to document the gel photo.